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Expression of a Self-Incompatibility Gene in a Self-Compatible Line of *Brassica oleracea*

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In cruciferous plants, self-pollination is prevented by the action of genes situated at the self-incompatibility locus or S-locus. The self-incompatibility reaction is associated with expression of stigma glycoproteins encoded by the S-locus glycoprotein (SLG) gene. Only a few cases of self-compatible plants derived from self-incompatible lines in the crucifer Brassica have been reported. In these cases, self-compatibility was generally ascribed to the action of single genes unlinked to the S-locus. In contrast, we report here a line of *Brassica oleracea* var *acephala* with a self-compatible phenotype linked to the S-locus. By means of both biochemical and immunochemical analyses, we showed that this self-compatible (*Sc*) line nonetheless possesses stigmatic SLGs (SLG-Sc) that are expressed with a similar spatial and temporal pattern to that described for the SLGs of self-incompatible Brassica plants. Moreover, the SLG-Sc products segregate with the self-compatibility phenotype in F_2 progeny, suggesting that changes at the S-locus may be responsible for the occurrence of the self-compatibility character. A cDNA clone encoding the SLG-Sc product was isolated, and the deduced amino acid sequence showed this glycoprotein to be highly homologous to the pollen recessive *S2* allele glycoprotein. Hence, self-compatibility in this Brassica *Sc* line correlates with the expression of a pollen recessive-like S allele in the stigma.

INTRODUCTION

In flowering plants, self-incompatibility mechanisms prevent self-fertilization by allowing the pistil to discriminate between self and nonself pollen grains (de Nettancourt, 1977). At the classic genetic level, the simplest and most common self-incompatibility systems encountered are controlled by a single multiallelic locus, the S-locus. In such systems, the self-incompatibility phenotype of the pollen is determined either by its own haploid genome, as in the Solanaceae, or by the diploid genome of the pollen-producing plant, as in the Cruciferae. The self-incompatibility response, that is, the rejection of self-pollen by the pistil tissues, occurs when the same S allele specificity as that present in the stigma, style, or ovary of the self-incompatible plant is also expressed by the pollen grain or pollen tube.

In the crucifer Brassica, the self-incompatibility phenotype has been correlated with the specific accumulation of glycoproteins in the stigma (Nasrallah and Nasrallah, 1984; Nasrallah et al., 1985a). Maximum synthesis of these glycoproteins occurs 1 day prior to anthesis and coincides with the acquisition by the stigma of the self-incompatibility character. Immunocytochemical studies have demonstrated that these glycoproteins are located at the site of inhibition of self-pollen tube growth in the papillar cell layer of the stigma surface (Kandasamy et al., 1989). The gene encoding these stigma glycoproteins, namely, the S-locus glycoprotein (SLG) gene, has been isolated and sequenced (Nasrallah et al., 1985b, 1987, 1988). Structural information concerning the SLG gene and its expression products has been gained from isolation and sequencing of cDNA clones encoding different SLGs (reviewed in Dickinson et al., 1992; Trick and Heizmann, 1992).

In *Brassica oleracea*, Lalonde et al. (1989) have shown that the SLG gene belongs to a multigenic family consisting of approximately a dozen related sequences. Among the various members of this family, another gene situated at the S-locus, the S-locus receptor kinase (SRK) gene encoding a putative receptor protein kinase, has been recently identified and characterized (Stein et al., 1991). Two other genes homologous to the SLG gene but not genetically linked to the S-locus are also expressed in the stigma, the S-locus–related (SLR1 and SLR2) genes (Lalonde et al., 1989; Boyes et al., 1991). The role played by these different genes in the self-incompatibility response is still unknown.

Although rare in the Brassicaceae family, a few cases have been reported of self-compatible plants derived from self-incompatible lines of Brassica. These include lines of *B. oleracea* var *acephala* (Thompson and Taylor, 1971), *B. oleracea* var *capitata* (Nasrallah, 1974), and *B. campestris* (Hinata

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et al., 1983; Hinata and Okazaki, 1986; Nasrallah, 1989). In all of these cases, self-compatibility was ascribed to a single suppressor (*sup*) or modifier (*mod*) gene unlinked to the self-incompatibility locus whose action seems to be strictly limited to the stigmatic tissue. Direct changes at the S-locus generating self-fertility alleles have never been demonstrated in these species. When *sup* genes are involved, a drastic reduction of SLG levels is observed in the stigma (Nasrallah, 1974, 1989). In the case of *mod* gene action, as reported in *B. campestris* (Hinata and Okazaki, 1986), self-compatibility might result from incorrect modification and/or activation of the SLG translational product (Nasrallah, 1989).

The study of plants presenting a genetic breakdown of self-incompatibility constitutes an original approach to understanding the role played by products of the S-locus genes in the self-incompatibility reaction. Here we report that a self-compatible (*Sc*) line of *B. oleracea* var *acephala* possesses stigmatic SLGs (SLG-Sc) whose spatial and temporal expression is similar to that described for self-incompatible Brassica plants. An immunochemical analysis of an F_2 population reveals that the SLG-Sc products segregate with the self-compatibility phenotype, suggesting that changes at the S-locus are responsible for the acquisition of the self-compatibility character. A cDNA clone encoding the SLG-Sc has been isolated, and the amino acid sequence deduced shows a high level of homology with that of the pollen recessive *S2* allele glycoprotein reported by Chen and Nasrallah (1990).

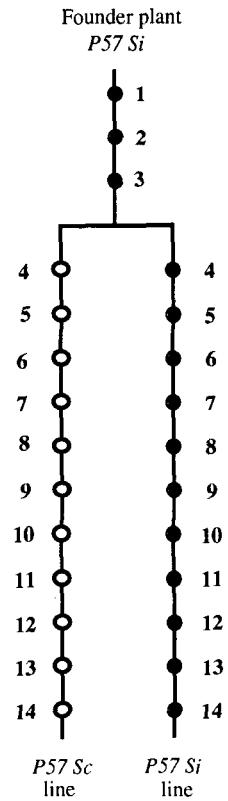

Figure 1. Pedigree of the *P57 Sc* and *P57 Si* Lines.

The founder plant, denoted *P57 Si*, was chosen from a population of self-incompatible *B. oleracea* plants of unknown S alleles. The *P57 Si* founder plant was self-pollinated on immature self-compatible flower buds to generate inbreds. Second and third generations of inbreds were produced by selfing one plant of at least 20 inbreds cultured at each new generation (indicated by bold-faced numbers). At the fourth generation of selfing, a self-compatible plant, denoted *P57 Sc*, was detected based on its high level of seeds set after self-pollination of mature stigmas. Progeny of the *P57 Sc* plant and of its self-incompatible sisters were shown to exhibit stable self-compatible and self-incompatible phenotypes, respectively. Dark and open circles correspond to self-incompatible and self-compatible phenotypes, respectively.

RESULTS

Genetics and Phenotype Description of the Self-Compatible Brassica Line *P57 Sc*

A self-incompatible line (*P57 Si*) of *B. oleracea* var *acephala* gave rise to a self-compatible line, denoted *P57 Sc*, after four generations of forced self-pollination. A pedigree of the two *P57* lines is represented in Figure 1. Stability of the self-incompatible and self-compatible phenotypes in these two lines was verified by inbreeding each line for 10 generations. A phenotypic analysis was undertaken to verify the self-compatibility of the *P57 Sc* line and to determine the pollen–stigma interaction phenotypes between the *Sc* and *Si* alleles in different pollination situations. The results of this analysis are presented in Figure 2. The level of self-compatibility of the *P57 Sc* plants was assessed by selfing buds at a stage where they would normally be self-incompatible, that is, from a few hours to 1 day prior to anthesis (Nasrallah et al., 1985a), and by comparing the yield of seeds with the yield from a compatible cross-pollination. Figure 2A shows the average number of seeds per silique estimated after selfing or crossing *P57 Sc* homozygotes. From a total of 42 flower buds treated per pollination experiment, an average of 15.1 seeds per fruit was obtained after selfing compared with 18.55 after crossing. In view of the comparable amount of seeds set after self- or cross-pollination, we assigned a self-compatibility phenotype to the *P57 Sc* line. Figures 2B to 2F show the pollen–stigma interaction phenotypes of the *P57* lines analyzed by microscopic observation of pollen tube growth following pollination. The self-compatibility and self-incompatibility phenotypes of the *P57 Sc* and *P57 Si* lines are illustrated in Figures 2B and 2C, respectively. Pollination of *P57 Sc* plants either by their own pollen or by pollen coming from sister plants led to the growth of several (generally more than 10) pollen tubes into the style. In contrast, usually zero or only a very low number of pollen tubes was observed after selfing *P57 Si* plants. We found that *P57 Sc* and *P57 Si* plants were reciprocally cross-compatible with regard to the dense pollen tube growth into the style (Figure 2D). A reciprocal

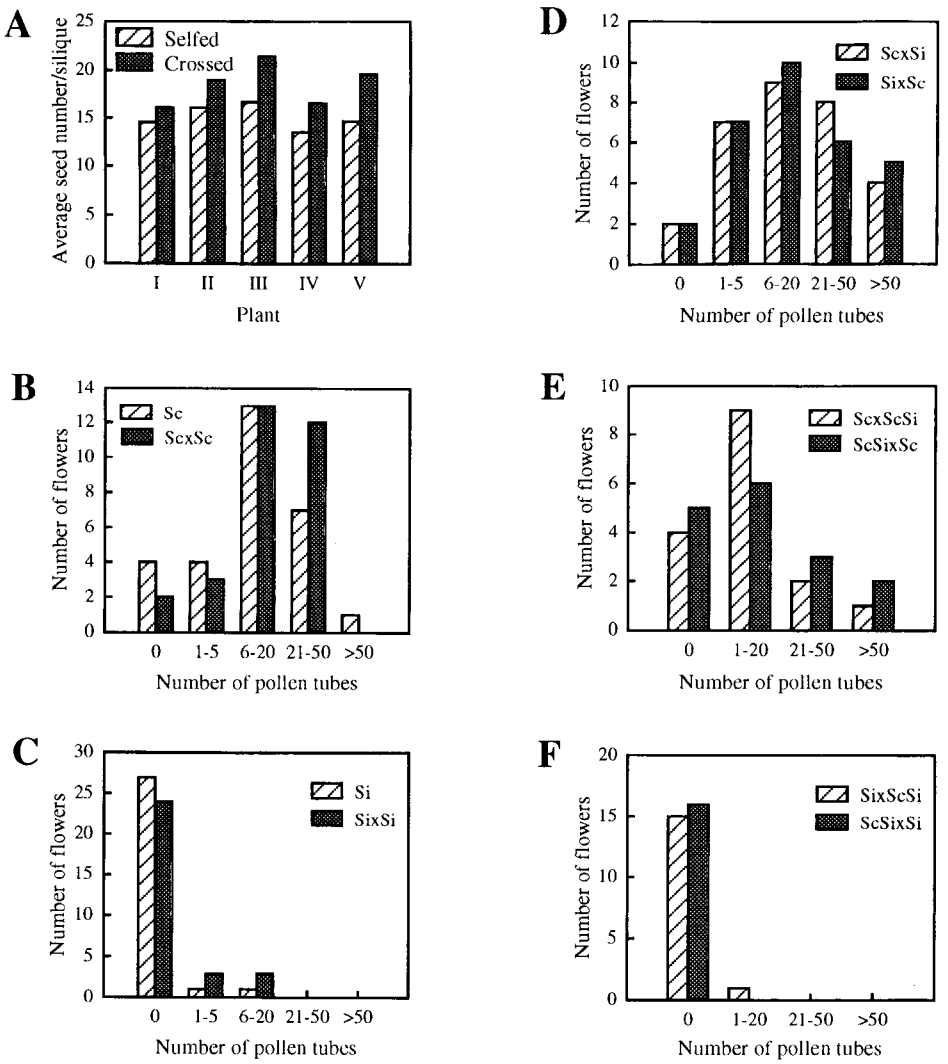

Figure 2. Phenotype Analysis of the *P57 Sc* and *P57 Si* Lines.

(A) Seed set after self- or cross-pollinating *P57 Sc* homozygotes. Five plants numbered from I to V were randomly chosen in a population of *P57 Sc* homozygotes. For each plant, nine flower buds at developmental stages ranging from −1 to −3 (see Methods) were either self-pollinated or cross-pollinated with an unrelated inbred line homozygous for a known S allele. The average number of seeds per silique was calculated for each plant. For plant IV, only six flower buds were tested. Hatched and stippled bars represent seed set after selfing or crossing, respectively.

(B) Number of pollen tubes growing into the pistil after self-pollinating *P57 Sc* homozygotes or cross-pollinating two *P57 Sc* plants. Bars represent the distribution of the number of pollen tubes growing in the pistil tissues for 30 flowers tested per pollination. Hatched and stippled bars, self-pollination (Sc) and cross-pollination (ScxSc), respectively.

(C) Number of pollen tubes growing into the pistil after self-pollinating *P57 Si* homozygotes or cross-pollinating two *P57 Si* plants. Bars represent the distribution of the number of pollen tubes growing in the pistil tissues for 30 flowers tested per pollination. Hatched and stippled bars, self-pollination (Si) and cross-pollination (SixSi), respectively.

(D) Number of pollen tubes growing into the pistil after reciprocal crosses between *P57 Sc* and *P57 Si* homozygotes. Bars represent the distribution of the number of pollen tubes growing in the pistil tissues for 30 flowers tested per pollination. Hatched bars, cross-pollination with *P57 Sc* homozygote as the female (ScxSi); stippled bars, cross-pollination with *P57 Si* homozygote as the female (SixSc).

(E) Number of pollen tubes growing into the pistil after reciprocal crosses between *P57 Sc* and *P57 Sc/Si* heterozygote. Bars represent the distribution of the number of pollen tubes growing in the pistil tissues for 16 flowers tested per pollination. Hatched bars, cross-pollination with *P57 Sc* homozygote as the female (ScxScSi); stippled bars, cross-pollination with *P57 Sc/Si* heterozygote as the female (ScSixSc).

(F) Number of pollen tubes growing into the pistil after reciprocal crosses between *P57 Si* and *P57 Sc/Si* heterozygote. Bars represent the distribution of the number of pollen tubes growing in the pistil tissues for 16 flowers tested per pollination. Hatched bars, cross-pollination with *P57 Si* homozygote as the female (SixScSi); stippled bars, cross-pollination with *P57 Sc/Si* heterozygote as the female (ScSixSi).

cross-compatibility relationship was also established between *P57 Sc* and the heterozygous plant *P57 Sc/Si* (Figure 2E), whereas reciprocal crosses between *P57 Si* and *P57 Sc/Si* were strongly incompatible (Figure 2F).

Identification, Characterization, and Location of SLGs in a Self-Compatible Brassica Line

To investigate whether the self-compatibility of the *P57 Sc* line could be attributed to a reduction or loss of the SLG gene expression, we first determined the SLG content of the two *P57* lines. Brassica SLGs are abundant glycoproteins that accumulate specifically in the stigmatic tissue of developing flowers and that have been identified by isoelectric focusing (IEF) in previous work (Nishio and Hinata, 1977; Nasrallah and Nasrallah, 1984). By a similar electrophoretic approach, we observed strong differences between the glycoprotein patterns of the two genotypes, as illustrated in Figure 3A. *P57 Sc* was characterized by two acidic/neutral glycoproteins, namely α and β, whereas *P57 Si* exhibited two basic glycoproteins, denoted δ and ε. A common band, γ, was detected in both stigma extracts, although less abundant in the *Si* line. In a previous study (Gaude et al., 1991), we reported that the γ protein was likely to be the product of the SLR1 gene in the *P57* lines. A significant decrease in the level of SLR1-like transcripts in the *P57 Si* line was also demonstrated by RNA gel blot analysis (Friry, 1991). Figure 3B shows that the *P57 Sc*– and *Si*–specific products were detected only in stigmatic extracts. The stigma-specific expression of these different glycoproteins indicates that they may be products of the SLG genes in the two lines.

To identify with more accuracy which of these proteins effectively may be products of the SLG genes, we obtained N-terminal amino acid sequence data for the most abundant specific proteins of the *P57* stigma extracts. The results of this analysis are shown in Table 1. The δ and ε proteins specific to the *P57 Si* line displayed identical N-terminal sequences that were homologous to that of SLG products of class I S alleles as defined by Nasrallah et al. (1991). In contrast, the β glycoprotein, which is specific to the *P57 Sc* line, was shown to possess two additional amino acid residues, Tyr and Val, within the N-terminal sequence. This N-terminal sequence is identical to that reported for the pollen recessive *S2* allele, which belongs to the class II S alleles (Nasrallah et al., 1991).

To check the *P57 Sc* genotype specificity of the β glycoprotein, we raised monoclonal antibodies (MAbs) against a synthetic peptide corresponding to the first 10 amino acid residues of the β protein. Figure 4 shows that the α and β bands in *P57 Sc* stigma proteins were recognized by the anti-β MAb 85-36-71, whereas no cross-reacting material was observed in the *P57 Si* extract. Furthermore, two other products (β′ and β″) with more basic pI values than the β protein were also faintly detected in the *P57 Sc* extract. To establish whether the *P57 Sc*–specific proteins accumulate during stigma development

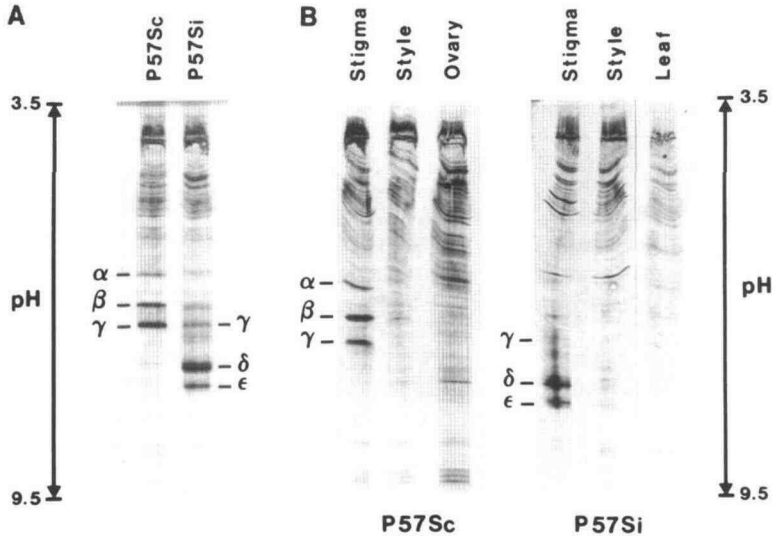

Figure 3. Identification of Putative SLGs in Stigmas of *P57 Sc* and *Si B. oleracea* Lines after Analytical IEF and concanavalin A–Peroxidase Glycoprotein Detection.

(A) Patterns of stigma glycoproteins from homozygous *P57 Sc* and *P57 Si* lines. The specific glycoproteins of the *P57 Sc* line (α, β), of the *P57 Si* line (δ, ε) and the common band γ are indicated.

(B) Glycoprotein patterns of sexual and vegetative tissues showing the stigma specificity of the α, β, γ, δ, and ε products from *P57 Sc* and *P57 Si* lines. Per deposit, 40 μg of total proteins was applied; the pH gradient was 3.5 to 9.5.

Table 1. N-Terminal Amino Acid Sequences of the Major Glycoproteins Purified from Stigma Extracts of *P57 Sc* (β and γ Proteins) and *P57 Si* (δ and ε Proteins) Lines and Comparison with the Peptide Sequences of SLG and SLR1 Proteins Predicted from the Corresponding cDNAs

Protein	pI	Amino Acid Sequence[a]	Number of Residues	Homology (%)[b] SLG-13	SLG-2	SLR1
β[c]	6.6	I YVNTLSSSE SLT I S	15	80	100	60
γ[c]	6.9	T - - NTLSPNE ALT I SSXKTL VSPGD	23	73.9	68	100
δ	8.15	I - - NTLSSTE SLT I S	13	100	80	69.2
ε	8.45	I - - NTLSSTE SLT I SXXXTL V	19	89.5	76.2	68.4
SLG-13[d]	—	I - - NTLSSTE SLT I SSNRTL VSPGN	23	—	84	73.9
SLG-2[d]	—	I YVNTLSSSE SLT I SSNRTL VSPGG	25	84	—	68
SLR1[d]	—	T - - NTLSPNE ALT I SSNKTL VSPGD	23	73.9	68	—

[a] One-letter notation is used for amino acids. X indicates positions in the sequence where residues could not be ascertained with accuracy. Gaps (–) have been introduced to provide optimal alignment between sequences. The underlined X or N residues constitute potential sites of *N*-glycosylation following the consensus sequence N-AA-S/T, where AA may be any amino acid residue except P as given in Struck and Lennarz (1980).

[b] Percentage of homology calculated in considering X as an asparagine.

[c] β and γ amino acid sequences are from Gaude et al. (1991). Sequence data were obtained by following the electrophoretic purification procedure described elsewhere (Gaude et al., 1991).

[d] The S13- and S2-locus-specific glycoproteins (SLG-13 and SLG-2) sequences were chosen as representative of class I and II S alleles, respectively, as defined by Nasrallah et al. (1991). The N-terminal amino acid sequences of the SLG-13 (Nasrallah et al., 1987), SLG-2 (Chen and Nasrallah, 1990), and SLR1 (Lalonde et al., 1989) proteins are predicted from the corresponding cDNAs.

in a similar manner to the SLGs of self-incompatible plants, stigma proteins extracted from different developmental stages of the flower were analyzed by immunoblotting. The result of this analysis is shown in Figure 5. MAb 85-36-71 recognized antigenic proteins early in the developing bud flowers, and these antigens accumulated throughout stigma maturation. Taking into account the N-terminal sequence data and the antibody reactivity exhibited by the specific stigma glycoproteins of the two *P57* lines, we will refer in the following discourse to the α, β, β′, and β″ proteins as SLG-Sc, and to the δ and ε proteins as SLG-Si.

MAb 85-36-71 was also used in an immunocytochemical study to determine whether an alteration in the location of the *P57* SLG-Sc in the female tissues could have led to the self-compatible phenotype. Figure 6 shows that as in normal self-incompatible Brassica plants, the SLG antigen was detected only in the papillar cell layer.

Segregation of *P57* SLG-Sc with the Self-Compatibility Phenotype

To determine whether the self-compatible phenotype was linked to the expression of the *P57 Sc*–specific glycoproteins, an F_2 population obtained by selfing a hybrid plant derived from a cross between *P57 Sc/Sc* and *S9S9* homozygotes was analyzed by compatibility/incompatibility phenotype determination and protein immunoblotting. Figure 7 illustrates the results of this analysis. Of 14 plants analyzed, only the plants expressing uniquely *P57 Sc* glycoproteins were self-compatible, whereas the remaining plants were all self-incompatible. Immunodetection of SLGs showed that these latter plants corresponded either to *S9S9* homozygotes or to *P57 Sc/S9* heterozygotes.

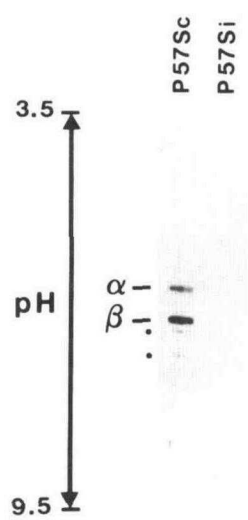

Figure 4. Immunocharacterization of the *P57 Sc* and *P57 Si* Lines with the Anti-β MAb.

Stigma proteins (10 μg per deposit) were separated on an IEF gel and electrotransferred onto nitrocellulose for immunodetection with anti-β MAb 85-36-71 (1:1000 dilution of ascitic fluid). Dots indicate the two less abundant proteins (β′ and β″) also detected by the antibody in the *P57 Sc* stigma extract. The pH gradient was 3.5 to 9.5.

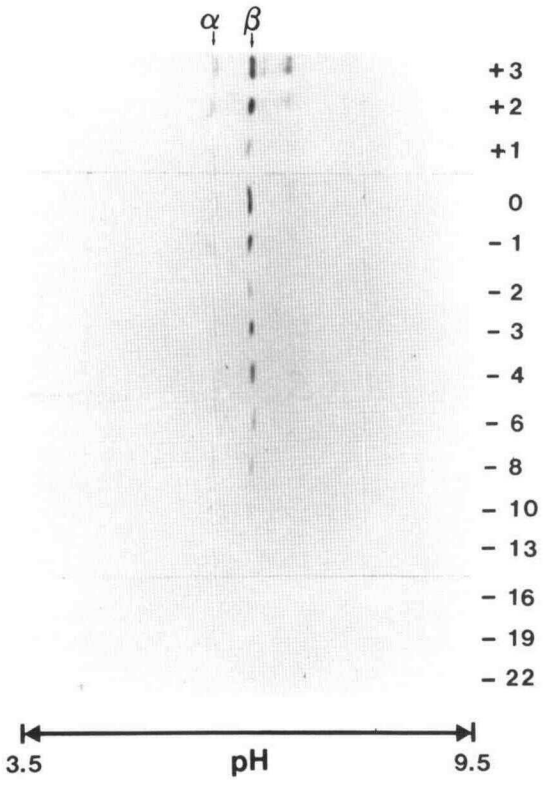

Figure 5. Appearance of *P57 Sc*–Specific Proteins during Stigma Development.

Stigma proteins from buds or flowers at various stages of development were analyzed as given in Figure 2, except that MAb 85-36-71 was diluted at 1:400. Proteins extracted from a single stigma at a defined stage of development were applied to each track. The numbers on the right designate the bud or flower developmental stage. Each bud or flower was given a number according to its position on the inflorescence: 0, flower at anthesis; positive numbers, open flowers after anthesis; negative numbers, buds prior to anthesis. The two major *P57 Sc*–specific glycoproteins α and β are indicated with arrows. The pH gradient was 3.5 to 9.5.

Isolation and Sequencing of a cDNA Clone Encoding the *P57* SLG-Sc

Our strategy for isolating the DNA sequences encoding the *Sc* glycoprotein was based on our knowledge of the N-terminal sequence of the β and δ/ε proteins. Oligonucleotides corresponding to these sequences were used as probes to screen cDNA libraries constructed with poly(A)$^+$ RNA from mature stigmas of *P57 Sc* and *Si* lines. Figure 8 demonstrates the specificity of these oligonucleotide probes, which allowed the isolation of eight *Sc* clones containing a β-like fragment and of two *Si* clones possessing δ/ε sequence homologies. A full-length *P57 Sc* clone (CG15) containing a 1.6-kb cDNA fragment of a length similar to that generally reported for SLG cDNA sequences was chosen for sequencing.

Figure 9 shows the deduced amino acid sequence of the CG15 cDNA insert aligned with published protein sequences of SLGs representative of class I and II S alleles (Nasrallah et al., 1991). The CG15 cDNA encodes a polypeptide of 445 amino acids that starts, like the other SLGs, with a signal peptide sequence of 31 residues. CG15 also has structural characteristics common to all SLGs (Nasrallah et al., 1987; Dickinson et al., 1992), for instance, the presence of 12 conserved cysteine residues in the C-terminal moiety of the protein and the existence of several potential sites of N-glycosylation. The sequence of the mature protein (1-414) has strong homology with SLGs of class II and particularly with the SLG-2B sequence described by Chen and Nasrallah (1990) (92.8% identity with SLG-2B, 88.2% with SLG-2A, and only 69.1% with SLG-13 of class I).

To identify the transcripts homologous to the cloned CG15 cDNA in *P57 Sc* stigmas, we performed an RNA gel blot analysis by hybridizing *P57 Sc* stigmatic RNA to the labeled CG15 cDNA. Figure 10 shows that two transcripts were detected: one at 1.6 kb with an intense hybridization signal and another one

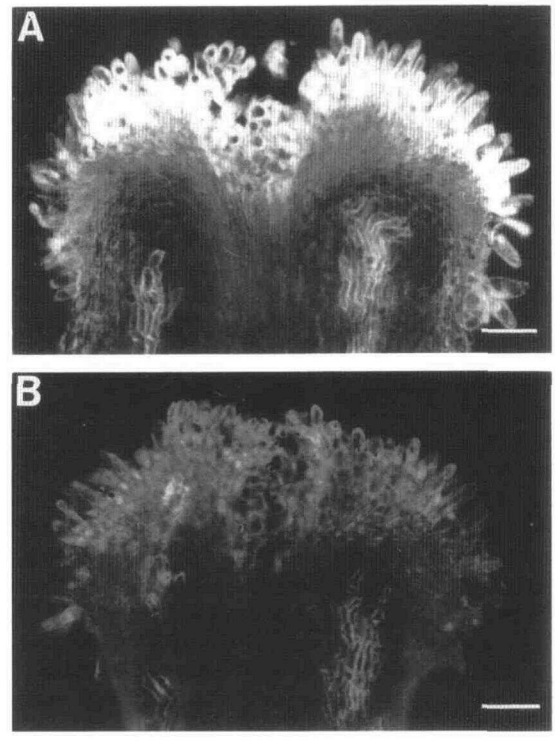

Figure 6. Immunolocalization of *P57 Sc*–Specific Proteins on Pistil Cryosections.

(A) Cryosection treated with anti-β MAb 85-36-71.
(B) Control with omission of the primary antibody. The fluorescence of vascular tissue and nuclei is nonspecific.
Bars = 0.1 mm.

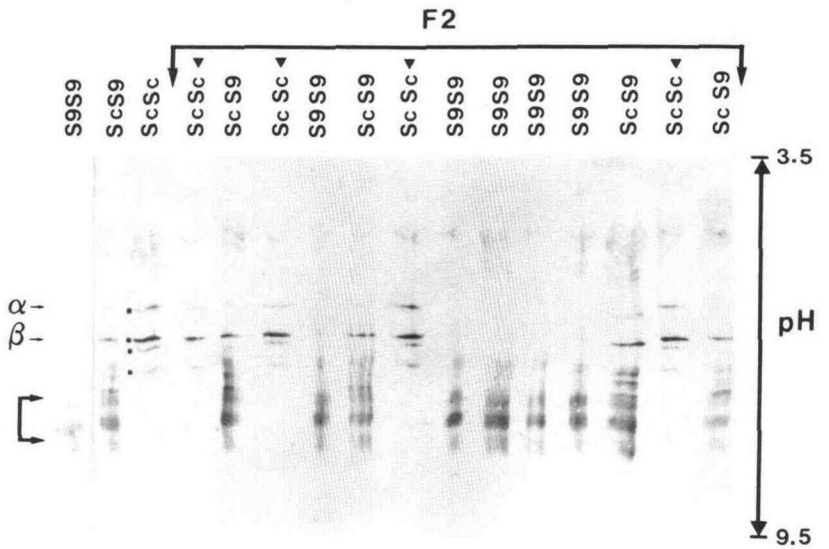

Figure 7. Analysis of an F_2 Population of Plants Segregating for the *Sc* and *S9* Alleles.

Proteins extracted from two stigmas per individual F_2 plant were subjected to IEF and electrotransferred onto nitrocellulose as given in Figure 4. The protein gel blot was treated simultaneously with an anti-SLG-9 mouse antiserum (1:1500 dilution) and with MAb 85-36-71 (1:400 dilution) to detect SLGs derived from either parent. The first three lanes on the left correspond to protein extracts from an *S9* homozygote (S9S9), *P57 Sc/S9* heterozygote (ScS9), and *P57 Sc* homozygote (ScSc), respectively. Protein patterns for 13 of 14 F_2 plants analyzed are illustrated and delimited by the bracket at the top. The bracket on the left indicates the complex of bands specific to the *S9* allele and dots indicate the four specific products of the *Sc* allele. Arrows indicate the position of the α and β glycoproteins. The genotype of each F_2 plant as defined by its immunodetection pattern is indicated on the top of the blot. The arrowheads indicate the four self-compatible F_2 plants; all the others rejected their own pollen or the pollen issued from the *S9S9* parent and accepted the *P57 Sc/Sc* pollen grains. The pH gradient was 3.5 to 9.5.

at 4.0 kb with a faint hybridization signal. Similar results were obtained, although with fainter hybridization signals, when a SLG-29 cDNA was used as a probe. The much stronger hybridization to the 1.6-kb transcript allowed us to consider that (1) the 1.6-kb signal corresponds to the CG15 transcript, and (2) the CG15 transcript is the SLG product expressed in the *P57 Sc* line.

DISCUSSION

Phenotype of the *P57* Lines

Analysis of seed set and pollen tube growth confirmed the self-compatibility character of the *P57 Sc* line and indicated a strong self-incompatibility reaction for the *P57 Si* line. The reciprocal cross-incompatibility relationship observed between *P57 Si* and the heterozygote *P57 Sc/Si* (Figure 2F) was indicative of a dominant interaction of the *Si* allele over the *Sc* allele in both pollen and pistil. The *S3* and *S9* alleles also showed a dominant relationship to *Sc* in heterozygous combination (I. Fobis, unpublished data). These observations with the data presented in this study suggest that the *Sc* allele is recessive in its action. Interestingly, the dominant interactions of *Si*, *S3*, or *S9* alleles on *Sc* were never associated with the absence of expression of the *P57 Sc*–specific glycoproteins in stigmas of heterozygous plants as demonstrated by immunostaining of protein gel blots (T. Gaude, unpublished data, and see Figure 7).

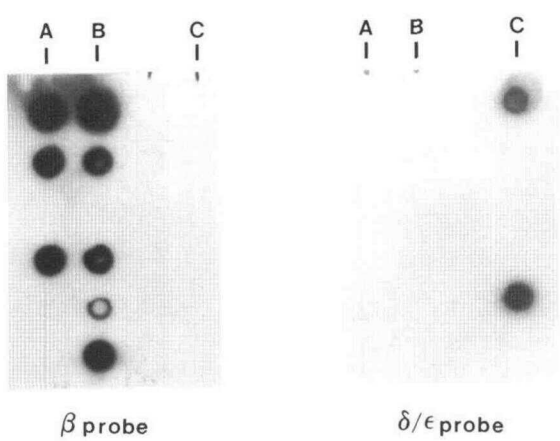

Figure 8. Isolation of the cDNA Clones Encoding the *Sc* Glycoproteins.

DNA dot blots of *P57 Sc* (lanes A and B) and *P57 Si* (lanes C) cDNA clones were hybridized with the β oligonucleotide (at left) or with the δ/ε probe (at right).

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                                                +1
CG15      -31  MKGVQNIYHHSYTFSFLLVFLVLILFHPALSIYVNTLSSSESLTISSNRT
SLG-2A         ================================================
SLG-2B         ================================================
SLG-13         =E==KKT=DI===L======F=====R==F==*=*=====T==========

CG15       20  LVSPGGVFELGFFKTLERSRWYLGIWYKKVPWKTYAWVANRDNPLSNSIG
SLG-2A     20  ===============P=G==Q==========SQ==============T====
SLG-2B     20  ===============P=G==========A================S====
SLG-13     18  =====N========TSS==========F=YR==V==========D==

CG15       70  TLKISGNNLVLLGQSNNTVWSTNITRGNVRSPVIAELLPNGNFVMRHSNN
SLG-2A     70  ======================L==E====================Y=S=
SLG-2B     70  ============S==T======L====A================I=====
SLG-13     68  ===========DH==KS=====V====E====V====D=======D==S

CG15      120  KDSSGFLWQSFDFPTDTLLPDMKLGYDLKTGRNRFLTSWKSSDDPSSGNF
SLG-2A    120  ==I==========================I===R=========T
SLG-2B    170  ==================E==================G=========
SLG-13    118  NNA=Q=======Y=======E==========L=======R=======DY

CG15      170  AYKLDLRRGLPEFILINTFLNQSVETQRSGPWNGMEFSGIPEVQGLNYMV
SLG-2A    170  T==I=TQ=========*=QGRY***=M=======================
SLG-2B    170  V====I==========Q====R=======================
SLG-13    168  S===E===*====Y=*SSGSF***RLH=======FRI=====D=K=S===

CG15      220  YNYTENSEEIAYSFHMTNQSIYSRLTISELTLD*RFTWIPPSWGWSLFWT
SLG-2A    216  ==========S=T==============V=DY==N*=L======RA==M===
SLG-2B    220  ========================V=======*=L======RD=======
SLG-13    213  ==F======A==T=L===N=F=======STGYFE=L==A=S=VV=NV==S

CG15      269  LPMDVCDPLYLCGSYSYCDLITSPNCNCIRGFVPKNPQQWDLRDGTQGCV
SLG-2A    265  ==T=============================================
SLG-2B    269  ==T=========================================R===
SLG-13    263  S=NHQ==MYRM==P=====VN===V====Q==R===R======IP=S==I

CG15      319  RTTQMSCSGDGFLRLNNMNLPDTKTATVDRTIDLKKCEERCLSDCNCTSF
SLG-2A    315  ============================I==V===============
SLG-2B    319  ================================M=V==============
SLG-13    313  =R=RL=======T=MK==K==E=TM=I=H=S=G==E==K=========A=

CG15      369  AIADVRNGGLGCVFWTGELVAIR*KYAVGGQDLYVRLNAADLGTCTG 414
SLG-2A    365  ========E==========E==*=F================       406
SLG-2B    369  ======================*=F================       410
SLG-13    363  =N==I==R=T===I=====ED==TYF=*D========A====V      404
```

Figure 9. Comparison of the Predicted Amino Acid Sequence Encoded by the CG15 cDNA with Those of SLG-2A, SLG-2B, and SLG-13.

One-letter notation is used to designate amino acids. Identical residues are indicated by a double line, and gaps (asterisks) have been introduced to provide optimal alignment between sequences. Asparagine residues constituting potential sites of N-glycosylation (seven sites) are indicated by filled circles; cysteines are marked with open circles. The CG15 and SLG-2B proteins share 92.8% sequence homology, although the CG15 sequence possesses two more potential N-glycosylation sites and is characterized by the addition of four amino acid residues at the C terminus that includes an extra cysteine. SLG-2A, SLG-2B, and SLG-13 predicted amino acid sequences are from Chen and Nasrallah (1990).

Expression of SLGs in the Self-Compatible *P57 Sc* Brassica Line

Taken together, our results demonstrate that the *P57 Sc* line defined as self-compatible with regard to its high levels of seed set on selfing does express SLGs. Like self-incompatible Brassica plants, the SLG-Sc products are abundant in mature stigmas, accumulate during flower development, and are located in the papillar cell layer of the stigmatic surface. Interestingly, the SLG-Sc products possess physical and structural features different from those identified in the *P57 Si* line from which the *P57 Sc* line is derived.

Following IEF analysis, the *P57* lines display two clearly distinguishable sets of glycoproteins: δ and ε for *P57 Si*, α and β for *P57 Sc*. For each line, these multiple compounds are likely to be glycoforms of a single gene product as was reported for Brassica SLGs following deglycosylation experiments and SDS-PAGE analysis (Isogai et al., 1987; Umbach et al., 1990). The identical N-terminal sequence of the δ and ε proteins and the reactivity of α, β, and the two other minor products in *P57 Sc* stigma proteins to the anti-β MAb are in agreement with such an assumption.

Based on structural differences among SLGs, on the existence of an epitope polymorphism, and on the strength of the self-incompatibility phenotype, Nasrallah et al. (1991) have defined two major classes of S alleles. Class I S alleles have strong self-incompatibility phenotype, present dominant interactions in heterozygous situations, and are exemplified by the *S6*, *S13*, *S14*, *S22*, *S29*, and *S63* alleles. Class II alleles, which include *S2*, *S5*, and *S15*, generally have a weaker incompatibility reaction and exhibit recessive and competitive interactions in pollen. The primary structure data obtained after N-terminal sequencing of β, δ, and ε proteins show that the two *P57* lines differ in their S allele content. According to the classification of Nasrallah et al. (1991), the S allele of *P57 Si* belongs to class I, whereas the S allele of *P57 Sc* is of class II (Table 1). This observation is confirmed by the nonreactivity of SLG-Si with the anti-β MAb, indicating an epitope polymorphism similar to the one described by Nasrallah et al. (1991).

This two-class distribution was also observed at the nucleic acid level. The strong identity between the amino acid sequence deduced from the CG15 cDNA and the SLG-2 sequences characteristic of class II alleles indicates that the *P57 Sc* SLG allele is one member of the class II alleles. The strict identity observed between the N-terminal amino acid sequence of the β glycoprotein and the corresponding region predicted for the CG15 protein supports the hypothesis that the CG15

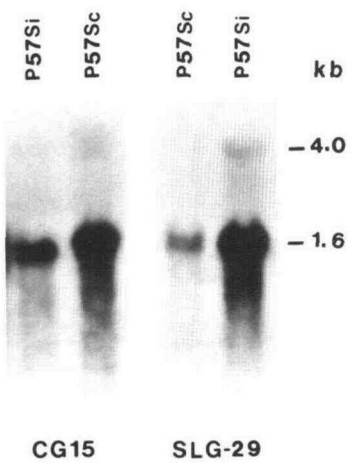

Figure 10. RNA Gel Blot Analysis of SLG Transcripts from *P57 Si* and *P57 Sc* Lines Hybridized with CG15 or SLG-29 Probes.

Transcripts of 1.6 and 4.0 kb were detected in the *P57 Sc* and *P57 Si* extracts with either CG15 or SLG-29 probes, respectively.

clone encodes the specific β glycoprotein of *P57 Sc*. Recently, another pollen recessive S allele, *S5*, has been cloned and sequenced (Scutt and Croy, 1992). The predicted CG15 polypeptide is even more homologous to SLG-5 (94.67%) than to SLG-2B (92.8%). The SLG-5 and CG15 amino acid sequences both contain an additional four residues at the C terminus (TCTG), which are lacking in all other sequences of the S gene family.

In a recent study (Stein et al., 1991), the gene encoding SLG-2B was found to correspond to the extracellular domain of a putative receptor protein kinase gene (SRK), which is located at the S-locus and consequently probably participates in the self-incompatibility reaction. In that study, the SLG-2B domain, used as a probe in RNA gel blot analysis of pistil poly(A)$^+$ RNA, hybridized to transcripts of 1.6, 3.0, and 4.1 kb. Only the 3.0- and 4.1-kb transcripts were assigned to the SRK gene, the smaller corresponding to a fully spliced SRK messenger and the larger to an unspliced SRK transcript. The highly abundant 1.6-kb transcript was shown to result from expression of the SLG gene. By analogy, when the CG15 cDNA was used as a probe on RNA from *P57 Sc* stigmas, an intense hybridization signal was obtained with a 1.6-kb transcript, whereas only a very faint signal was detected at 4.0 kb (Figure 10). The 1.6-kb transcript probably corresponds to the SLG-Sc gene and the 4.0-kb transcript to an unspliced transcript of the SRK-Sc gene. The absence of signal at 3.0 kb may be due to our experimental conditions or to a difference of genotype. However, further analysis with an SRK probe is needed to establish whether the *Sc* line expresses multiple SRK mRNA species.

Origin of the Self-Compatibility Phenotype

The presence of products of the SLG gene in stigmas of the *P57 Sc* line and their expression with a similar spatial and temporal pattern to that described for the SLGs of self-incompatible plants exclude the involvement of *sup* genes in the manifestation of the self-compatibility phenotype of *P57 Sc*. Furthermore, expression of the self-compatibility phenotype corresponds with the presence of the SLG-Sc in stigmas of *P57 Sc/Sc* homozygotes in the F_2 progeny. This observation was confirmed by the analysis of another population of F_2 progeny (20 plants) obtained by selfing a *P57 Sc/S3* heterozygote plant (I. Fobis, unpublished data). These results suggest that changes at the S-locus or the action of a modifier gene closely linked to this locus may be responsible for the occurrence of the self-compatibility character. However, because only small populations of F_2 plants were analyzed, it is still possible that the gene controlling the self-compatibility character could be several map units away from the S-locus.

Our present experimental data, therefore, contrast with the previously reported instances of self-compatible Brassica lines (see Nasrallah, 1989), where the phenotype has been shown to be associated with loci unlinked to the S-locus. An S-locus–linked self-compatibility phenotype could presumably arise as a result of either direct mutation of S-locus gene(s) (either SLG and/or SRK or as yet unidentified S-locus genes), or as a result of the mutation of an S-locus–linked *mod* gene, and, hence, an indirect effect on expression of gene(s) at the S-locus. One of the most striking points of our study is that the self-compatible and the self-incompatible lines express two different SLGs. However, because of the high degree of polymorphism of the S gene family, it is not possible to identify specific modifications to the CG15 gene that may be causing the self-compatible phenotype. Note that the reciprocal nature of the compatibility phenotype suggests that the expression of S-locus genes in male tissues must also be affected. However, little is known about the male components of the self-incompatibility reaction (see Dickinson et al., 1992). Whatever the origin of these modifications of the S-locus products, the result is a breakdown in the self-incompatibility response, due, for example, to a mismatch between interacting S-locus–encoded molecules or to an inefficient signal transduction mediated by an altered SRK gene product.

Two hypotheses can be suggested to explain the occurrence of the self-compatible character from the self-incompatible phenotype during the breeding program. The first hypothesis proposes that the *P57 Si* line was fixed before the occurrence of self-compatibility. In this case, we may speculate that during forced self-pollination of the *P57 Si* line, dramatic changes occurred at the S-locus, perhaps as a result of gene conversion events involving other members of the S multigenic family, and leading to the appearance of the *Sc* allele. This hypothesis does not involve alterations to modifier genes because the self-compatibility character is uniquely dependent on S-locus mutations. The second hypothesis proposes that the *P57 Si* line was not fixed before the occurrence of self-compatibility. In this case, we must imagine that the starting plant was actually a heterozygote *Si/Sc* and that heterozygous *Si/Sc* plants were chosen as parents for each new generation of selfing, generating an F_2 population each time with increasing homozygosity. Only at the fourth generation would one of the *Sc/Sc* plants have exhibited a self-compatible phenotype allowing its detection by the breeder.

To distinguish between these two hypotheses, restriction fragment length polymorphism and random amplified polymorphic DNA analyses have been undertaken to determine the level of genomic homology between *P57 Sc* and *Si* lines. Preliminary results have detected no differences between *P57 Si* and *Sc* genomes apart from the different SLG genes (P. Heizmann and V. Delorme, unpublished data). The direct transition from the *Si* to the *Sc* allele could probably have been demonstrated using our molecular probes to identify the SLG components of plants belonging to the third and fourth generations of selfing. This plant material is unfortunately no longer available.

To conclude, the various experimental approaches undertaken in this study demonstrate that like self-incompatible *B. oleracea* plants, the self-compatible *P57 Sc* line expresses products of the SLG gene. Moreover, as supported by the genotypic characterization of the F_2 progeny plants, the self-compatibility character seems to segregate with the presence

of the SLG-Sc in stigmas of *P57 Sc/Sc* homozygotes. This observation excludes the involvement of *sup* genes unlinked to the S-locus to explain the occurrence of the self-compatible *P57 Sc* plants. Rather, we suggest that the breakdown of self-incompatibility might have been the result either of dramatic changes occurring at the S-locus, leading to the appearance of the *Sc* allele homologous to the pollen recessive *S2* allele, or alternatively may have involved a *mod* gene closely linked to the S-locus and/or active only in the absence of a dominant S allele.

METHODS

Plant Material

A self-incompatible line, denoted *P57 Si*, and a self-compatible line, namely *P57 Sc*, of *Brassica oleracea* var *acephala*, were kindly provided by Dr. Véronique Ruffio from the INRA station of Rennes, France. The *P57 Sc* line was derived from a self-compatible plant that occurred after four generations of forced self-pollination of the *P57 Si* line. Each line was homozygous for an undetermined S allele that we arbitrarily defined as *Si* and *Sc* for the self-incompatible and self-compatible lines, respectively. A self-incompatible line of *B. oleracea* var *acephala* homozygous for the *S9* allele was also used and was obtained from Dr. Toby Hodgkin at the Scottish Crop Research Institute, Invergowrie, Dundee, Scotland. Plants were grown in the field and were maintained by self-pollination of immature self-compatible buds.

Staging of Floral Development

Individual buds or flowers along the inflorescence were numbered starting with 0 for the flower at anthesis and assigning positive values to open flowers following the flower at anthesis, and negative values to buds prior to anthesis. For instance, the bud and the flower flanking the flower at anthesis corresponded to −1 and +1 stages, respectively.

Compatibility/Incompatibility Phenotype Determination

Phenotype determination was performed on flowers at anthesis, with nondehiscent anthers, or on floral buds at developmental stages ranging from −1 to −3. Stigmas were hand pollinated using pollen grains collected the day of pollination. Inflorescences were covered by an insect proof cellophane envelope to prevent contamination of pollinated flowers with stray pollen. Pollen tube growth in the pistil tissues was monitored microscopically using decolorized aniline blue as a fluorescent stain (Martin, 1959).

Sixteen to 30 flower buds on a total of three plants were tested per pollination experiment. For each pistil analyzed, the number of pollen tubes penetrating the style was counted 24 hr after pollination and the pistil was scored, according to the value found, into one of five classes: 0, no pollen tube detected; 1–5, from one to five pollen tubes; 6–20, from six to 20 pollen tubes; 21–50, from 21 to 50 pollen tubes; >50, more than 50 pollen tubes. In this assay, when most pistils ranged in the 6–20 class or in a higher class, the interaction was defined as compatible, whereas the absence of growth or only a low number of pollen tubes (1–5 class) was indicative of an incompatible interaction. The self-incompatibility or self-compatibility phenotype of F_2 plants was determined by performing reciprocal crosses with homozygous *S9* and *P57 Sc* tester lines and by selfing each plant (five flowers used per pollination). In all experiments, the pollen quality and stigma receptivity of tested plants were controlled by performing cross-pollination with an unrelated inbred line homozygous for a known S allele.

Protein Extraction and Electrophoretic Analysis

Proteins were extracted, separated on isoelectric focusing (IEF) polyacrylamide gels, and the glycoproteins were detected as described previously (Gaude et al., 1991). Following IEF separation, proteins were electrotransferred onto a nitrocellulose membrane as previously described (Gaude et al., 1991), except that the transfer buffer used was an aqueous solution of 0.7% acetic acid.

Antibody Production

For the production of anti-β glycoprotein antibodies, mice were immunized with the peptide IYVNTLSSSEC cross-linked to ovalbumin by *m*-maleimidobenzoic acid *N*-hydroxysuccinimide ester (Van Regenmortel et al., 1988). Monoclonal antibodies (MAbs) were obtained by following the procedure described by Lane et al. (1986). Ascites were formed by injecting 10^6 to 10^7 hybrid cells into mice. Mouse antiserum against S-locus glycoprotein (SLG)-9 was raised by intraperitoneal injections of a high-performance liquid chromatography–purified SLG-9 protein.

Immunodetection on Protein Blots

Following transfer of proteins onto nitrocellulose, membranes were incubated in TBST-BSA buffer (10 mM Tris-HCl, pH 8.0, containing 150 mM NaCl, 0.05% Tween 20, and 1% BSA) for 1 hr. The BSA/Tween-blocked membranes were then reacted with primary antibodies (anti-β monoclonal or anti–SLG-9 polyclonal) diluted with TBST-BSA for 1 hr. After probing, membranes were washed 3 × 5 min in buffer without BSA (TBST) and incubated with secondary antibodies (alkaline phosphatase–conjugated goat anti–mouse IgG [Promega, Madison, WI]) diluted 1:7000 in TBST-BSA for 30 min. Protein blots were again rinsed 3 × 5 min in TBST. Antigen–antibody complexes were visualized in a color reaction medium of 0.33 mg mL^{-1} nitro blue tetrazolium chloride, 0.165 mg mL^{-1} 5-bromo-4-chloro-3-indolyl phosphate, in 100 mM Tris-HCl, pH 9.5, containing 100 mM NaCl and 5 mM $MgCl_2$.

Immunohistochemistry

Pistils of the *P57 Sc* line were embedded in OCT compound (Tissue Tek, embedding medium for frozen tissue specimens; Miles Inc., Elkhart, IN) and directly frozen in liquid nitrogen. Ten-micrometer thick cryosections were treated for 2 hr at room temperature in a 1:100 dilution of both the primary MAb and the goat anti–mouse secondary antibody conjugated to fluorescein isothiocyanate (Caltag, San Francisco, CA) in TBST-BSA.

cDNA Library Construction, DNA Sequencing, and RNA Gel Blot Analysis

Two cDNA libraries from mature stigmas of *P57 Sc* and *P57 Si* lines were constructed in the plasmid pUEX1 (Amersham International,

Buckinghamshire, England) and differentially screened through two stages of hybridizations: BS29-2 and BS29-1 probes (Trick and Flavell, 1989) were used in the first round to select SLG and S-locus-related (SLR)1 gene sequences, respectively. The second round was performed on SLG clones from *P57 Sc* (total of 17 clones) and *P57 Si* (seven clones) with two synthetic oligonucleotides corresponding to the first five N-terminal amino acids of the β protein (equivalent to β oligonucleotide) and the δ/ε proteins (equivalent to δ/ε oligonucleotide). The β oligonucleotide was made without any degeneracy according to the published recessive sequence (positions 92 to 108, CG ATC TAT GTC AAC ACT) of the SLG-2 cDNA (Chen and Nasrallah, 1990). The δ/ε oligonucleotide (TCG ATC AAC ACT TTG TCG) was deduced from the corresponding region of sequences SLG-6 (Nasrallah et al., 1987) and SLG-29 (Trick and Flavell, 1989). A full-length CG15 clone (1.6 kb) was subcloned into M13mp18 and sequenced by the dideoxy nucleotide chain termination method (Sanger et al., 1977) using the Sequenase system (U.S. Biochemicals). For RNA gel blot analysis, 10 μg of total RNA from the two *P57* lines was denaturated in formamide/formaldehyde, fractionated on formaldehyde-agarose gels (Sambrook et al., 1989), blotted onto Hybond N^+ filters, alkali fixed (0.05 M NaOH for 5 min), hybridized with in vitro–labeled CG15 or SLG-29 (BS29-2 from Trick and Flavell, 1989) probes, and washed at low stringency (42°C in 0.5 × SSC [1 × SSC is 0.15 M NaCl, 0.015 M sodium citrate], 0.1% SDS).

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